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ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 90
1 August 1952

COLD HEMAGGLUTININS AND FROSTBITE*

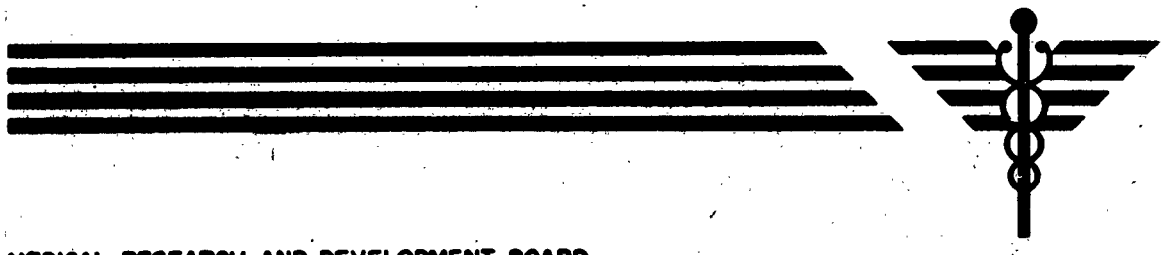
with

APPENDIX

on

VARIABLES IN THE DETERMINATION OF COLD HEMAGGLUTININS

*Subtask under Environmental Physiology, AMRL Project No. 6-64-12-028, Subtask (8), Cold Injury Studies.



MEDICAL RESEARCH AND DEVELOPMENT BOARD
OFFICE OF THE SURGEON GENERAL
DEPARTMENT OF THE ARMY

REPORT NO. 90

COLD HEMAGGLUTININS AND FROSTBITE *

with

APPENDIX

on

VARIABLES IN THE DETERMINATION OF COLD HEMAGGLUTININS

Report of the AMRL Cold Injury Research Team

Exercise Snowfall

January-February 1952
Camp Drum, New York

by

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from

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ABSTRACT

COLD HEMAGGLUTININS AND FROSTBITE

OBJECT

To study the relationship between cold hemagglutinins and various factors concerned with susceptibility to cold injury.

RESULTS AND CONCLUSIONS

No significant difference in cold agglutinin titers was found between the frostbite cases and the control subjects.

Negro subjects had significantly higher titers than White subjects. The incidence of frostbite was approximately four times as high among Negroes as among Whites at Exercise Snowfall.

No significant correlation was found between cold agglutinin titer and age.

Among White subjects men with Group O blood had significantly lower cold agglutinin titers than men with blood of the other three groups. Men with Group A and Group B blood had titers intermediate between those of Group O and Group AB blood.

White subjects with a history of frequent upper respiratory infections had significantly higher cold agglutinin titers than those without such history. The data, although statistically not significant, were suggestive in the case of Negro subjects.

Infantrymen had significantly higher cold agglutinin titers than support personnel. The incidence of frostbite was highest among infantry troops. There may, therefore, be a relationship between cold agglutinins and frostbite. A rise in cold agglutinin titers was found immediately after the maneuver and a marked fall in titers was found 8 months after the maneuver in White subjects.

No correlation was found between cold agglutinin titers and temperature of habitual environment.

RECOMMENDATIONS

Additional investigation seems indicated to evaluate the relationship between frostbite and cold hemagglutination.

The possible interactions between blood group agglutinins and cold agglutinins also deserve further study.

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COLD HEMAGGLUTININS AND FROSTBITE

I. INTRODUCTION

The in vitro agglutination of erythrocytes in contact with serum at low temperatures has been observed in both animals and humans since 1903, when Landsteiner (13) and Biffi (4) first described this phenomenon. Landsteiner's studies formed the basis for subsequent work in this field. He demonstrated the dependence of the agglutination on low temperature with dispersion on rewarming, prepared solutions of agglutinin in normal saline solution, and showed that the agglutinin is present in the globulin fraction of the serum. This latter demonstration was confirmed by Clough and Richter (7), followed by Stats et al (22) who showed, by electrophoretic mobility studies, that the agglutinin is present in the gamma globulin fraction.

Certain distinguishing characteristics of cold hemagglutination have been well established and are summarized as follows: 1) Red blood cell agglutination by serum containing cold agglutinins is best demonstrated at 0° to 5°C., rarely above 25°C. and almost never at 37°C.; 2) Such agglutination is reversed by warming to 37°C. and reappears on cooling. No apparent damage to the red cells occurs as this process is repeated; 3) The serum can be exhausted by repeated absorption in the cold; 4) The agglutinin is not inactivated by heating to 56°C. for 30 minutes; 5) The agglutinin survives cold storage with only slight decrease in strength for periods ranging from 3 weeks to a year or more; 6) The agglutinin is active against human cells of any group and against animal cells of many unrelated species.

In normal healthy humans the occurrence of cold hemagglutinins has varied from 14.6% of 49 "normals" (11) to 47% of 238 sera drawn for routine Wasserman tests (1), differences which could possibly be accounted for by differences in technique (see Appendix). Although cold agglutinins have been associated with a variety of clinical conditions foremost among which are primary atypical pneumonia (10, 11, 12, 18, 23), trypanosomiasis (6, 9, 27), and acquired hemolytic anemias (2, 10, 19), it is of present interest to note its association with peripheral vascular disturbances such as Raynaud's phenomenon (2, 3, 8, 15, 21), two cases of which resulted in gangrene of the tips of the extremities. In the case reported by Stats and Bullowa (21), unilateral hemoglobinemia was demonstrated following exposure of one forearm to cold, and intravascular hemagglutination, discontinuity of the blood stream and slowing of blood flow in conjunctival vessels followed irrigation of the conjunctival sac with iced saline solution.

The possible relationship between the presence of cold hemagglutinins and the occurrence of frostbite and other forms of local cold injury was mentioned by Parker (17) in association with trench foot, by Lange, Weiner and Boyd (14) in observations on intravascular clumping of red cells in tissues exposed to cold, by Stats and Wasserman (23) in observations suggesting that individual tolerance to cold may depend on the titer of cold agglutinins and by Weiner (25) in a demonstration showing first, the more frequent presence of cold agglutinins in human frostbite cases (74.8% of 115 cases) when compared with unselected controls (40.3% of 308 cases), and second, the observation that higher titers of cold agglutinin seemed to appear more frequently in the frostbite than control cases. Weiner found no rise in titer of humans and animals exposed to cold and suggested a pathogenetic role in frostbite for high titer cold agglutinins due to intravascular clumping of red blood cells, slowing of blood flow and occlusion of small vessels following exposure to cold.

The undertaking of a large scale cold weather maneuver by the Army and Air Force (Exercise Snowfall, Camp Drum, New York, January-February, 1952) provided an opportunity to study troops under simulated combat conditions. A team was organized by the Army Medical Research Laboratory at Fort Knox for the purpose of evaluating the relationship of cold hemagglutinins to cold injury.

Operations began on 8 January 1952. From 8 January to 6 February, the training phase of the Exercise, cold hemagglutinin titers and associated data (see below) were obtained. Cold injury casualties occurring during this period and during the tactical phase of the Exercise (9-16 February) were received in a cold injury ward staffed by members of the Cold Injury Research Team. A follow-up study on some of the troops previously examined was performed during the week of 18 February. Further follow-up studies of cold agglutinins were accomplished at Fort Campbell, Kentucky, in April and in October 1952.

The present study was an attempt 1) to obtain added data on the relationship between cold hemagglutinins and frostbite and, 2) to show if other associated factors such as age, type of military duty, etc., have any bearing on the presence of cold agglutinins with respect to frostbite.

II. METHODS AND MATERIALS

A. Subjects

Since frostbite is primarily a disease of the combat soldier this study, for the most part, was limited to troops of the various

combat organizations taking part in the Exercise. Included also, however, were certain support elements (such as signal, transportation, engineer, ambulance and medical units) whose mission involved at least some exposure to cold weather. In addition, cold agglutinin titers were determined on 126 of the 148 cases of frostbite that occurred during the Exercise. Table 1 gives the breakdown, by organization, of all White and Negro subjects, both control and frostbite, from whom cold agglutinin titers and other data were obtained.

TABLE 1

COLD HEMAGGLUTININ DETERMINATIONS BY ORGANIZATION IN WHITE AND NEGRO CONTROL SUBJECTS AND FROSTBITE CASES

	Total Maneuver Strength		Control Subjects		Frostbite Cases	
	White	Negro	White	Negro	White	Negro
3rd Armored Cavalry Regiment	1847	831	953	489	1	14
278th Regimental Combat Team	3090	223	1460	105	13	7
11th Airborne Division	11361	1652	3202	469	52	30
306th Logistical Command	2582	1161	818	132	4	2
Others and Organization Unknown			27	2	0	3
Totals	18880	3867	6460	1197	70	56
Grand Totals	22747		7657		126	

The figures for total maneuver strength were obtained from the General Plan of the Exercise. The figures for relative White and Negro total maneuver strength were obtained from various regimental and battalion headquarters and are close approximations but not exact figures. Not included in the table are 64 subjects of Mongolian extraction and 57 subjects of undetermined race. This makes a grand total of 7904 subjects from whom cold agglutinin titers and other data were obtained. Thus, the sample consists of 28.8% of the total strength and is closely representative with regard to relative White and Negro strength.

Control subjects were tested both in the pre-maneuver period and at varying intervals up to 8 months following the maneuver. Studies at the 6 to 8 week follow-up and 8 month follow-up were conducted on members of the 11th Airborne Division at Fort Campbell, Kentucky, who had previously been tested while engaged in the cold weather exercise at Camp Drum. Table 2 gives the number of troops tested before maneuver and the numbers re-tested at varying intervals after the maneuver.

TABLE 2
COLD HEMAGGLUTININ DETERMINATIONS ON WHITE AND NEGRO CONTROL SUBJECTS

Time of Test	Number of Tests		
	White	Negro	Total
Before Maneuver	6460	1197	7657
1 to 3 Days After Maneuver	857	160	1017
6 to 8 Weeks After Maneuver	1165	180	1345
8 Months After Maneuver	539	56	595
Total Tests Performed	9021	1593	10614

Of the 126 frostbite cases on whom cold hemagglutinin determinations were performed only 61 were tested before they received their cold injury. Other determinations were performed at various intervals after injury (Table 3).

TABLE 3
COLD HEMAGGLUTININ DETERMINATIONS PERFORMED ON FROSTBITE CASES

Time of Test	Number of Tests
Before Injury	61
1 to 3 Days After Injury	22
1 Week After Injury	65
2 to 4 Weeks After Injury	35
5 to 7 Weeks After Injury	19
8 to 10 Weeks After Injury	34
Total Tests Performed	236
Total Frostbite Cases Tested	126

B. Procedures

1. Collection of Data

Data obtained on each subject at the initial interview included:

a. Name
b. Service Serial Number
c. Rank
d. Organization
e. Age
f. Race
g. State in which subject had lived most of his life.
h. Blood group (from individual identification tag).
i. History, within the preceding year, of frequent upper respiratory infections (a minimum of 4 attacks of "cold", bronchitis, etc.)
j. History of pneumonia within the preceding year.

A venous blood specimen for cold hemagglutinin determination was then drawn from each subject. The data were recorded on code sheets devised to facilitate transfer of the information to punched cards for machine sorting and tabulating.

2. Determination of Cold-Hemagglutinin Titers

The general method of determining cold agglutinin titers in this study was similar to the methods employed in other laboratories (11, 18, 19, 23).

In brief, the method consisted simply in placing a suspension of erythrocytes in contact with various dilutions of the unknown serum and incubating overnight at just above freezing temperature. The mixtures were observed the following morning to determine the highest dilution of the serum in which definite agglutination of the erythrocytes could be seen. In view of the wide variation in technique and in results often found in reports from different laboratories (1, 10, 22), the technique used in the present study is reported in detail.

Venous blood (approximately 5 to 7 ml.) from the antecubital vein was drawn and placed in a chemically clean test tube (13 x 100 mm.). Twenty such samples from as many donors filled one test tube rack which was then placed in a water bath at 37°C. for 1 hour. The rack was then removed and the tubes were placed in a centrifuge and spun at 2000 R.P.M. for 30 minutes.

The serum was then decanted into a similarly numbered tube with special care to avoid transfer of red cells. Serial dilutions were then prepared as follows: The required number of tubes to be used for titration was placed in a Wasserman rack. Omitting tube 1, 0.5 ml. of 0.85% sodium chloride was placed in each tube thereafter. Five-tenths of a milliliter quantities of serum were added to tubes 1 and 2. The serum and saline in tube 2 were mixed by repeatedly drawing up small portions of the mixture into a 1 ml. pipette. Five-tenths of a milliliter of this serum-saline mixture was then transferred to tube 3 and mixed with the already present saline as in tube 2. This procedure was repeated in each tube thereafter. Five-tenths of a milliliter of solution was removed from the last tube and discarded. A final quantity of 0.5 ml. of serum-saline mixture remained in each tube at this point.

To each tube 0.5 ml. of a 1% suspension of freshly prepared type "O" red cells (group "O" red cells confirmed microscopically) was added. The 1% suspension of red cells in normal saline was prepared daily as follows. Twenty-two and two-tenths milliliters of

venous group "O" blood was drawn into a flask containing 500 ml. of saline and mixed by inverting the flask four or five times. This mixture was then centrifuged at 1500 R. P. M. for 10 minutes. The saline was decanted following which more saline was added to the centrifugate. This procedure was repeated three times. The final saline-red cell centrifugate was re-suspended in 1, 000 ml. of saline.

After the addition of red cells each test tube (of the series of serum dilutions) contained 1 ml. of the mixture. The solution was thoroughly mixed by gentle shaking. The final serum dilution in the first tube was then 1:2; in the second tube 1:4; etc.

The mixtures of erythrocyte suspension and serum dilutions were then placed overnight in a refrigerator (65 cu. ft.) the temperature of which was kept between 2° and 4°C. After at least 16 hours of refrigeration, the tubes were observed immediately upon removal from the refrigerator. A 2 inch magnifying lens and a strong light was used for reading. Each tube was shaken 3 times with a moderate snap of the wrist before reading. The highest dilution in which definite agglutination could be seen was recorded as the titer of cold hemagglutination. The tests were read by the same individual throughout the entire study in as nearly a uniform manner as was possible.

Titer values, for purposes of statistical evaluation, were recorded as the tube number of the highest positive dilution, as follows:

Titer	0	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Recorded as Tube No.	0	1	2	3	4	5	6	7	8

C. Frostbite Cases

A total of 148 cases of frostbite occurred during the pre-maneuver and maneuver periods. These were classified by degree of injury according to criteria similar to those used by Orr and Fainer (16):

First degree - 60
Second degree - 83
Third degree - 5

The criteria used were as follows:

First degree: Numbness, erythema, swelling and superficial desquamation of the involved part, possibly stinging or burning pain and swelling after rewarming.

Second degree: Vesiculation of the skin, involving only partial thickness of the skin and not extending into subcutaneous tissue.

Third degree: Involvement of entire thickness of skin, extending into varying depths of subcutaneous tissue. Vesicles may or may not appear.

Fourth degree: Damage to the entire thickness of the part including bone, usually resulting in loss of the part. Few or no vesicles are seen.

Injury of lesser degree usually was present proximal to the most severe injury. It was sometimes not possible to classify third degree cases accurately until vesicles had broken and ulceration had developed. No fourth degree cases occurred during this study.

III. RESULTS

A. Cold Hemagglutinin Titers and Frostbite

No significant difference in cold agglutinin titers was found between the frostbite cases and the control subjects (Tables 4 and 5) although it should be borne in mind that the frostbite cases in this study were, for the most part, very mild (96.6% first or second degree).

TABLE 4

COLD HEMAGGLUTININ TITERS IN WHITE AND NEGRO FROSTBITE AND CONTROL SUBJECTS

Titer (Tubes)	Number of White Subjects		Number of Negro Subjects	
	Control	Frostbite	Control	Frostbite
0 (o)	2734	27	328	15
1 (1:2)	1924	21	354	20
2 (1:4)	1259	12	339	17
3 (1:8)	249	4	80	2
4 (1:16)	139	3	40	0
5 (1:32)	119	2	41	0
6 (1:64)	33	1	13	1
7 (1:128	2	0	2	1
8 (1:256)	1	0	0	0
Total	6460	70	1197	56
Mean Titer	1.0	1.2	1.4	1.3
Standard Deviation	±1.19	±1.40	±1.35	±1.32

TABLE 5

COMPARISON OF MEAN TITERS IN TABLE 5

	Pairings	Number of Men	Mean Titer (Tubes)	Standard Deviation	P (t-test)
1	White Control	6460	1.0	±1.19	>0.2
	White Frostbite	70	1.2	±1.40	
2	Negro Control	1197	1.4	±1.35	>0.4
	Negro Frostbite	56	1.3	±1.32	
3	White Control	6460	1.0	±1.19	<0.001
	Negro Control	1197	1.4	±1.35	

B. Cold Hemagglutinin Titers and Race

Tables 4 and 5 also show the significant difference in cold agglutinin titers found between White and Negro subjects. Whether this finding has any bearing on the higher frequency of frostbite among Negroes found in this study (Table 6) is an open question. Others also have shown Negroes to have a higher incidence of frostbite (16, 25). No satisfactory explanation has been established. Lack of appropriate data in this study precluded the possibility of evaluating such factors as education, training, motivation and hygiene, all of which have been implicated (16). The possibility of an inherent racial predisposition must also be considered.

TABLE 6

THE INCIDENCE OF FROSTBITE AMONG WHITE AND NEGRO TROOPS AT EXERCISE SNOWFALL

	White	Negro	Total
Total Maneuver Strength*	18880	3867	22747
Frostbite Cases	81	67	148
Incidence per thousand	4.29	17.33	6.51

*Major organizations, enlisted men.

C. Cold Hemagglutinin Titers and Age

The ages of the subjects in this study ranged from 17 to 55 years (98.4% were between 18 and 35 years). Within this limited age range no significant correlation was found between cold agglutinin titer and age among the White control subjects (Table 7).

TABLE 7

COLD HEMAGGLUTININ TITER AND AGE IN WHITE CONTROL SUBJECTS

	Age	Cold Agglutinins Titer (Tubes)
Range	17 to 55	0 to 8 (1:256)
Mean	22.8	1.0
Standard Deviation	±3.44	±1.112
Number of Men	6456*	
Correlation	-0.019	
P	>0.1	

*Age of 4 subjects unknown.

D. Cold Hemagglutinin Titers and Blood Group

An interesting relationship was found between blood group and mean cold agglutinin titers (Table 8). Among the White control subjects men with Group O blood had the lowest mean titer (0.9 tubes), men with Group AB blood had the highest (1.2 tubes) and men with Group A and Group B were intermediate (1.1 tubes). If the blood groups could have been relied upon, these findings might suggest that the presence of alpha agglutinin or beta agglutinin (in Groups B and A respectively) depresses the cold agglutinin titer about equally while the presence of both alpha and beta agglutinins (in Group O) depresses the cold agglutinin titer even further. It should be noted that the blood group of each individual was determined by examining his identification tag. Surveys have indicated that as many as 14.9% or more are erroneously tagged (20). For this reason no statistical evaluation of the above findings was attempted.

TABLE 8

COLD HEMAGGLUTININ TITERS BY BLOOD GROUP IN WHITE AND NEGRO CONTROL SUBJECTS

		Blood Group			
		O	A	B	AB
White	Number of Men*	3110	2197	603	219
	Mean Titer (Tubes)	0.9	1.1	1.1	1.2
	Standard Deviation	±1.14	±1.21	±1.19	±1.36
Negro	Number of Men*	643	272	198	45
	Mean Titer (Tubes)	1.4	1.6	1.4	1.2
	Standard Deviation	±1.35	±1.32	±1.38	±1.30

*Blood group unknown for 331 white subjects and 39 negro subjects.

E. Cold Hemagglutinin Titers and Respiratory Infections

Table 9 compares cold agglutinin titers in subjects with and without a positive history of frequent upper respiratory infections (four per year) or pneumonia within the preceding year. White subjects with a history of frequent upper respiratory infections were found to have significantly higher titers than those without such history. The other pairings showed no significant differences although the figures suggest that Negro subjects with frequent upper respiratory infections might show higher titers.

TABLE 9

COLD AGGLUTININ TITERS AND HISTORY OF FREQUENT UPPER RESPIRATORY INFECTIONS AND PNEUMONIA IN WHITE AND NEGRO CONTROL SUBJECTS

		History of Freq. U.R.I.	No History of Freq. U.R.I.	History of Pneumonia	No History of Pneumonia
White	Number of Men*	1728	4725	1208	5245
	Mean Titer (Tubes)	1.1	1.0	1.0	1.0
	Standard Deviation	±1.23	±1.17	±1.19	±1.19
	P - (t-test)	<0.02		>0.4	
Negro	Number of Men	358	839	171	1026
	Mean Titer (Tubes)	1.5	1.4	1.5	1.4
	Standard Deviation	±1.45	±1.30	±1.46	±1.32
	P (t-test)	>0.1		>0.9	

*No data on medical history for 7 white control subjects

F. Cold Hemagglutinin Titers and Type of Military Duty

For purposes of comparison control subjects were divided into three groups according to the degree of exposure to climatic factors their type of duty was likely to involve in average "combat" situations. The three groups, in order of severity of average exposure, are as follows:

1. Infantry (278th Regimental Combat Team, 188th Airborne Infantry Regiment, 503rd Airborne Infantry Regiment and 511th Airborne Infantry Regiment)

2. Armor and Artillery (3rd Armored Cavalry Regiment, 11th Airborne Division Artillery, 710th Tank Battallion)

3. Support (306th Logistical Command, Umpire Group, Others)

Table 10 shows the comparisons of the cold agglutinin titers obtained in subjects of the various groups. It may be seen that both in White and Negro subjects infantrymen had significantly higher titers than support troops. Among White subjects infantrymen had significantly higher titers than armor and artillery men while among Negroes armor and artillery men had significantly higher titers than support personnel. It appears, from these findings, that increasing degrees of exposure to cold weather are associated with higher cold hemagglutinin titers. The overall incidence of frostbite among troops engaged in this maneuver seems to follow the same pattern (Table 11), infantry troops having the highest incidence per thousand (7.45) and support troops the lowest (4.01).

TABLE 10

COLD AGGLUTININ TITERS BY TYPE OF MILITARY DUTY IN WHITE AND NEGRO CONTROL SUBJECTS

	Pairing	White				Negro			
		Number of Men*	Mean Titer (Tubes)	Standard Deviation	P (t-test)	Number of Men	Mean Titer (Tubes)	Standard Deviation	P (t-test)
1	Infantry	3800	1.1	±1.23	<0.001	390	1.5	±1.41	>0.9
	Armor & Artillery	1815	0.9	±1.09		673	1.5	±1.33	
2	Infantry	3800	1.1	±1.23	<0.001	390	1.5	±1.41	<0.001
	Support	825	0.9	±1.13		134	1.0	±1.13	
3	Armor & Artillery	1815	0.9	±1.09	>0.2	673	1.5	±1.33	<0.001
	Support	825	0.9	±1.13		134	1.0	±1.13	

*Organization unknown for 20 white control subjects

TABLE 11

INCIDENCE OF FROSTBITE BY TYPE OF MILITARY DUTY

Type of Military Duty	Number of Men	Frostbite Cases	Incidence per Thousand
Infantry	12703	90	7.45
Armor and Artillery	6931	43	6.20
Support	3743	15	4.01
Total	22747	148	6.51

G. Changes in Cold Hemagglutinin Titers After the Maneuver

Table 12 shows the cold agglutinin titers obtained at various intervals after the maneuver and compares them to the pre-maneuver titers. Among the White subjects a significant rise in titer seemed to occur immediately after the maneuver following which there was a

TABLE 12

COLD HEMAGGLUTININ TITERS AT VARIOUS INTERVALS AFTER MANEUVER IN WHITE AND NEGRO CONTROL SUBJECTS

		Before Maneuver (January)	vs. 1-3 Days After Maneuver (February)	vs. 6-8 Weeks After Maneuver (April)	vs. 8 Months After Maneuver (October)
White	Number of Men	6460	857	1165	539
	Mean Titer (Tubes)	1.0	1.4	1.0	0.2
	Standard Deviation	±1.19	±1.24	±1.18	±0.54
	P (t-test)	---	<0.001	>0.7	<0.001
Negro	Number of Men	1197	160	180	56
	Mean Titer (Tubes)	1.4	1.5	1.5	0.4
	Standard Deviation	±1.34	±1.32	±1.49	±1.06
	P (t-test)	---	>0.8	>0.6	>0.7

fall to pre-maneuver levels 6 to 8 weeks later and a marked fall 6 months later (8 months after maneuver). Among Negro subjects no statistically significant changes were observed although the drop in titers in the few subjects tested at the 8 month interval was suggestive. Whether these findings were due to seasonal variation or were, possibly, regressions of changes which occurred during the pre-maneuver and maneuver periods remains to be determined.

H. Cold Hemagglutinin Titers After Frostbite

Cold hemagglutinin determinations performed on the frostbite cases at various intervals after injury are summarized in Table 13.

TABLE 13

COLD HEMAGGLUTININ TITERS AT VARIOUS INTERVALS AFTER INJURY IN WHITE AND NEGRO FROSTBITE CASES

Time of Test	White		Negro	
	Number Tested	Mean Titer (Tubes)	Number Tested	Mean Titer (Tubes)
Before Injury	42	1.0	19	1.3
1 to 3 Days After Injury	17	1.8	5	1.0
1 Week After Injury	29	0.9	36	1.3
2 to 4 Weeks After Injury	16	1.1	19	0.6
5 to 7 Weeks After Injury	13	0.5	6	1.5
8 to 10 Weeks After Injury	18	0.5	16	0.7

Since the numbers of tests involved are small and since no consistent pattern is apparent no statistical analysis of these figures is presented.

I. Cold Hemagglutinins and Climate

An effort was made to determine whether a relationship existed between cold hemagglutinin titer and the temperature of the locality in which the individual had lived most of his life. Product-moment correlation coefficients were computed between the cold agglutinin titers and the "minimum", "average" and "maximum" temperatures of the states in which the individual, by his own statement, had lived most of his life. Temperature data were obtained from Weather Bureau Technical Paper No. 9 (24) and were based on an average observation period of 59 years. For purposes of this analysis the mean daily minimum temperatures during the month of January were used as the "minimum" temperatures, the mean daily maximum temperatures during the month of July were used as the "maximum" temperatures and the mean annual temperatures were used as the "average" temperatures. Where temperatures from more than one observation station within a state were available a population-weighted average was used for that state. The results of this analysis (Table 14) indicated that no significant correlation existed between cold agglutinin titer and temperature of "habitual" residence.

TABLE 14

THE RELATION OF COLD HEMAGGLUTININ TITERS TO 'MINIMUM', 'AVERAGE' AND 'MAXIMUM' ENVIRONMENTAL TEMPERATURES IN WHITE AND NEGRO CONTROL SUBJECTS

	White Subjects (6384)*				Negro Subjects (1193)*			
	Cold Agglutinin Titers	vs. 'Min.' Temp.	vs. 'Aver.' Temp.	vs. 'Max.' Temp.	Cold Agglutinin Titers	vs. 'Min.' Temp.	vs. 'Aver.' Temp.	vs. 'Max.' Temp.
Range	0 to 8 (Tubes)	-4.0°F to +54.0°F	39.6°F to 72.1°F	75.8°F to 102.7°F	0 to 8 (Tubes)	+12.8°F to +54.0°F	45.0°F to 72.1°F	75.8°F to 102.7°F
Mean	1.0	24.3°F	53.6°F	84.2°F	1.4	29.2°F	57.7°F	86.3°F
Standard Deviation	±1.19	±8.90°	±7.08°	±3.91°	±1.35	±10.77°	±5.49°	±2.89°
Product-Moment Correlation (r)	---	-0.003	+0.002	+0.007	---	+0.052	+0.045	-0.029
P	---	>0.8	>0.8	>0.5	---	>0.05	>0.1	>0.3

*Habitual residence unknown for 76 white subjects and 4 negro subjects.

A similar analysis of the frostbite cases also failed to reveal any significant correlation.

It is of interest to note that the mean temperatures of the control subjects (from Table 14) are almost identical to the corresponding mean temperatures of the frostbite cases (Table 15). This

indicates that the frostbite cases, on the average, did not come from warmer climates than did the control subjects. Thus, the earlier implications of Orr and Fainer (16) that individuals living in cold climates may be less susceptible to cold injury than individuals living in warm climates is not supported by this type of analysis for the limited number of mild frostbite cases presented here.

TABLE 15

ENVIRONMENTAL TEMPERATURES OF FROSTBITE CASES AND CONTROL SUBJECTS

Mean Temperatures	White		Negro	
	Frostbite Cases	Control Subjects	Frostbite Cases	Control Subjects
'Minimum'	24.0°F	24.3°F	29.8°F	29.2°F
'Average'	53.1°F	53.6°F	57.2°F	57.7°F
'Maximum'	83.6°F	84.2°F	85.7°F	86.3°F

IV. DISCUSSION

Although the relationship between frostbite and cold hemagglutinins previously reported (25) was not confirmed by this study the reader is again reminded that the frostbite cases in this study were relatively few in number and mild in degree, whereas the cases cited in the above reference were all third and fourth degree. The finding of both higher incidences of frostbite and higher cold agglutinin titers in Negro subjects as compared to White subjects and in infantrymen as compared to support personnel suggest that a link of some sort does exist between frostbite and cold agglutinins. A more precisely controlled study is needed to evaluate this possible relationship.

The higher titers of cold agglutinins found among men with a history of "frequent upper respiratory infections" suggest that many of those men may have suffered unrecognized mild episodes of atypical pneumonia, a disease which regularly produces a rise in cold agglutinin titers.

The possibility that blood group agglutinins may have a neutralizing action against cold agglutinins is interesting. The question of whether cold agglutinins, in turn, have any action on blood group agglutinins immediately comes to mind and might be of practical importance. This is a problem which seems to demand further study.

The lack of support in this study for the concepts of acclimatization and "accustomization" by no means disproves their possible existence as important factors in the epidemiology of frostbite. Experimental work (5) has shown that in rabbits and rats acclimatization can

be developed by exposure to cold for only six weeks and disappears in three or four weeks. Since no data were available in this study concerning the exposure of the subjects in the months immediately preceding the Exercise no conclusions as to the existence of short term acclimatization could be drawn. On the other hand, the results of this study seemed to deny the existence of any long lasting acclimatization on the part of individuals who had lived most of their lives in cold climates.

Because of the paucity of data no conclusions are presented regarding the effect of frostbite on the cold agglutinin titers. However, the rise in titer of the White control subjects immediately following the maneuver favored the conclusion that exposure to low temperatures did have an effect. This was further supported by the marked drop in titers noted in subjects tested 8 months later, after a hot summer in Southern Kentucky. The higher titers found in infantry troops, who were presumably more exposed to the elements, also supported this concept.

V. CONCLUSIONS

Cold hemagglutinin titers were higher in Negro subjects than in White subjects. The incidence of frostbite was higher among Negroes than among Whites.

Cold hemagglutinin titers were higher among infantry troops than among support troops. Frostbite was more frequent among infantrymen.

This suggests a possible relationship between cold agglutinins and frostbite.

The incidence of frostbite was not higher among men who had lived most of their lives in warm climates as compared to those who had lived most of their lives in cold climates, nor was there any difference in their cold agglutinin titers.

"Frequent upper respiratory infections" seemed to be associated with higher cold agglutinin titers.

There seemed to be a relationship between blood group and cold agglutinins.

VI. RECOMMENDATIONS

Further study is recommended to evaluate the practicability of

using cold agglutinins and other related factors as an aid in classifying military personnel as to their ability to function in cold weather operations.

The possibility of a mutually antagonistic action existing between cold agglutinins and blood group agglutinins deserves further investigation.

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APPENDIX

VARIABLES IN THE DETERMINATION OF COLD HEMAGGLUTININS

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VARIABLES IN THE DETERMINATION OF COLD HEMAGGLUTININS

I. INTRODUCTION

The present methods for the analysis of cold hemagglutinins are fraught with many difficulties affecting the validity of the results obtained. The test is widely employed in hospitals and research laboratories primarily in connection with its use as a diagnostic aid in primary atypical pneumonia. In spite of an impressive array of published articles on cold agglutination during the past decade and its almost universal acceptance as a useful laboratory test, the methods employed for its determination are almost as numerous as the investigators utilizing it. (For a few methods, see Table 1.)

TABLE 1

PUBLISHED METHODS FOR DETERMINATION OF COLD AGGLUTININS

TECHNIC	U.S.ARMY (9)	KABAT & MEYER (8)	PLATT & WARD (11)	FINLAND ET AL (4-7)	RESP.DIS.COMM. (1)
SERUM-					
Clotting	Room Temp.	---	Room Temp. or 37°C	Room Temp. or 37°C	---
Storing	Refrig. before use	---	---	Refrig. 2-3 days at 5-10°C	---
RED BLOOD CELLS					
Storage of washed cells	Fresh cells daily	---	Use 1 day old cells	Use 2-4 day old cells	Fresh cells daily
Type	'O' (1%)	Patient's own cells (1-2%)	'O'	'O'	'O' (0.2%)
Washing temp.	Room temp.	37°C	37°C	---	---
REFRIGERATOR					
Temperature	0-5°C	In ice water in icebox overnight	Below 5°C	0-5°C	4°C
Mixing after initial cooling	No	Yes. In first few hours	---	---	---
READING					
Temperature	Remove from refrig.	In ice water while reading	In ice water while reading	In ice water	Remove from icebox
Dispersal of RBC	'Bottom is flicked'	---	Invert tube 3 times	Invert tube 3 times	'Gentle shaking'
Rewarming	Yes. Read in 2 hrs.	---	---	Yes. Read in 2 hrs.	Yes. Read in 30 min.

In the past year attention has been focused on the cold agglutinin test with respect to its possible use as an indicator of susceptibility to frostbite (14). To this end, approximately 13, 000 tests have been performed on animals, normal humans, and frostbite subjects. In the course of these studies, and during discussions with other workers also engaged in frostbite research, it became strikingly apparent that small deviations in technic might have a significant effect on the results and that, in the final analysis, such results were derived from a subjective appraisal of the test tube agglutination of red blood cells previously agitated into suspension.

From July 1951 to July 1952 five large groups of experiments involving the use of the cold agglutinin test were performed by members of the Army Medical Research Laboratory both at Fort Knox and in Japan and Korea. Each group of studies involved certain changes in the method of determining cold hemagglutinins. It has since become apparent that the over-all usefulness of combining such large groups of data from this test is limited by the variables in each method which might affect the results. It is the purpose of this report to delineate such variables which might affect the cold agglutinin test and to give certain experimental data which was designed to analyze such variables.

II. DISCUSSION OF METHODS

In general, methods for determining cold agglutinins include the following procedures:

1. Collection and clotting of whole blood.
2. Preparation of red cell suspension.
3. Dilution of serum.
4. Refrigeration of serum-red cell mixture.
5. Reading of agglutinations.

A. Collection and Clotting of whole blood

Venous blood, preferably from the fasting subject, is drawn into chemically clean test tubes in amounts varying from 5 to 10 milliliters. It is then allowed to clot either at room temperature or at 37°C. for at least one hour. The wide thermal range of occasional specimens of cold agglutinin may result in the loss of some of the agglutinin at 25°C. (11, 12). Turner (13) states, however, that clotting

at room temperature (25°C.) does not result in a detectable loss in antibody titer compared to clotting at 37°C. After complete clotting has occurred the serum may be removed for titration or it may be stored for as much as several days at 5°C. without losing potency (2, 3, 10, 12).

Plasma, obtained by adding oxalate or heparin to whole blood at the time of collection, may be used in the determination of cold agglutinins. Table 2 shows the similarity of values obtained using serum and plasma. (Method involving manual shaking of tubes was used.)

TABLE 2

PLASMA AND SERUM COLD AGGLUTININS

A. NORMALS	PLASMA (Heparin)	SERUM
H.V.	1:256	1:256
S.S.	1:8	1:16
R.L.	1:8	0
M.E.	1:8	1:8
W.S.	1:8	0
J.A.	1:8	1:8
D.M.	1:8	1:8
L.P.	1:64	1:64
C.D.	1:64	1:64
R.W.	1:128	1:64
B. PRIMARY ATYPICAL PNEUMONIA		
I.O.	0	1:2
C.V.	1:4	0
R.P.	1:8	1:64
W.T.	0	1:2
E.S.	1:16	1:4
S.M.	1:256	1:256
F.H.	1:128	1:128
F.M.	1:128	1:128
G.B.	1:8	1:8

P = >0.6 (no significant difference)

B. Preparation of Red Blood Cell Suspension

Though several authors use suspensions of the patient's own red cells in performing titrations, the use of type "O" red cells is now standard practice. For laboratories which do a relatively large number of cold agglutinin tests each day or week the preparation of a type "O" red cell suspension which can be used on all the tests eleminates the excess time required to process each patient's red cells.

Intra-group reactions are also obviated with a type "O" red cell suspension, though the possibility of sub-group reactions has not been eliminated.

Pooled red cells may be used although the dangers of sub-group reactions within a pool may be obviated by using one donor throughout the testing period.

The red cells are washed, usually three times, in saline at room temperature although some authors (8, 11) recommend washing at 37°C. It has been the custom of this laboratory to wash red cells at room temperature. The speed and duration at which the red cells may be centrifuged from a saline suspension during washing varies somewhat with the laboratory. Cells centrifuged for 10 minutes at 1500 R.P.M. for three successive washings do not appear to give substantially different readings from those centrifuged for one hour at 2000 R.P.M. (See Table 3).

TABLE 3

EFFECT OF VARIABLE CENTRIFUGATION OF RED CELL SUSPENSION ON COLD AGGLUTININ TITERS

CENTRIFUGATION			PATIENTS (TITERS)		
Group	R.P.M.	Minutes	S. S.	J. C.	M. V.
I	1500	10 min., wash 10 min., wash 10 min., wash	1:32	1:8	1:64
II	2000	10 min., wash 10 min., wash 30 min., wash	1:32	1:8	
III	1500	10 min., wash 10 min., wash 30 min., wash			1:64
IV	2000	30 min., wash 30 min., wash 1 hour, wash	1:16	1:8	1:32

Finland et al (4-7) claim that washed red cells stored from 2 to 4 days are more sensitive and of greater value in reading true titers than freshly washed suspensions. Others disagree (1, 9) using only fresh red cell suspensions daily. It has been the practice of this laboratory to use fresh red cell suspensions daily.

The concentration of red cells in the saline suspension is usually 1 to 2 per cent, affording easy visibility, although some methods involve the use of lower concentrations for greater sensitivity (4-7).

C. Dilution of Serum

Great care must be taken in the pipetting of serum dilutions since a residue of small amounts of undiluted serum may line the top of the pipette and be subsequently washed into the terminal dilutions.

Chemically clean dry pipettes must be used for each determination. Adequate mixture of serum and saline in each tube is usually accomplished by repeatedly sucking up the serum-saline mixture to a given point in the pipette and then discharging it into the solution. (A mechanical pipette might eliminate errors.)

D. Refrigeration of Serum-Red Cell Mixture

After thorough mixing of the serum-saline dilutions to which the red cell suspension has been added, the test tube racks are placed in a refrigerator. The temperature of the refrigerator is kept usually between 0^{o} and 5^{o}C; some investigators (8) prefer to keep the racks immersed in ice water while being refrigerated.

It is usually feasible to leave the racks in the refrigerator overnight, removing them for reading in the morning.

A few investigators (8, 12, 14) prefer to re-shake the racks after they have been in the cold for several hours in order to bring the red cells into close contact with the cold agglutinin in the serum.

Though some warming of the average 6 cubic foot refrigerator will occur when racks are repeatedly removed for reading, such small degrees of warming will not significantly affect the final results if the test tubes are read immediately upon removal.

E. Reading the Titer

The titer readings appear to be subject to considerable variability, and will be dealt with in some detail.

1. Temperature of tube readings

Although some authors (4-7, 8, 11) recommend placing the racks in ice water before reading, it is apparent that prompt reading of the tubes eliminates this need. The temperature elevation in the interval between removal of the rack from the refrigerator and reading is negligible if the rack is read immediately after removal.

2. Dispersal of RBC

The cold agglutination of red cells is known as a "soft" aggregate (11), and as such is easily dispersed by heat and mechanical agitation. Methods of dispersing these aggregates so that they are readily visible vary from "gentle shaking" (1), the "bottom (of the test tube) is flicked" (9) to "inverting the test tube three times" (4-7, 11).

Table 4 illustrates the variability in readings obtained on three aliquots of a single titration. Fifteen subjects were used. Each titration was read by each of three observers whose readings were performed without knowledge of the other readers' values. Observers I and II shook the tubes gently with a very slight wrist motion sufficient only to stir the red cells from the bottom of the tube into suspension. Observer III shook each tube with 3 vigorous "snaps" of the hand. It is apparent that Observer III, by increased mechanical agitation, obtained lower titers and a greater number of negative titers than the first two observers whose readings were statistically similar, largely due to similarities in shaking the tubes. Such subjective differences might be resolved by using a mechanical agitator which would shake the tubes a constant number of times with constant force, thus facilitating reproducible readings. The tubes could then be removed and read without further agitation. Table 5 illustrates the constancy of titers obtained with a mechanical agitator. Optimum speeds for best visibility of agglutinins were used. Agitation for 4 seconds, with an over-all excursion of 1 inch and an excursion speed of 350 per minute was found to give easily reproducible results*

TABLE 4

INDIVIDUAL VARIATION IN READING COLD AGGLUTININ TITERS

CASE	OBSERVER I	OBSERVER II	OBSERVER III
1	1:4	0	0
2	1:32	1:16	1:4
3	0	1:2	0
4	1:4	1:2	0
5	1:16	1:8	0
6	0	1:4	1:4
7	1:512	1:256	1:512
8	1:8	1:16	1:16
9	0	1:2	0
10	1:8	1:8	1:4
11	1:8	1:8	0
12	1:4	1:4	0
13	1:8	1:8	0
14	1:8	1:8	1:2
15	1:16	1:32	0

*A Cenco-Meinzer Laboratory Shaker (Central Scientific Company, Chicago, Illinois) with variable speeds was used.

TABLE 5

COLD AGGLUTININ TITERS OBTAINED WITH MECHANICAL SHAKER

Sample	PATIENTS		
	S. S.	A. J.	H. V.
1	1:4	1:16	1:16
2	1:2	1:16	1:16
3	1:4	1:16	1:16
4	1:4	1:16	1:32
5	1:4	1:16	1:16
6	1:2	1:32	1:16

Shaker Excursion: 1 inch
To and Fro Frequency: 350 excursions per minute
Duration of Shaking: 4 seconds

Product-moment correlation of above data for reader reliability $r = +0.94$
Significance of product-moment correlation $p = <0.0001$

3. Rewarming

Most investigators prefer to verify the presence of true cold agglutination by re-reading the tests after incubation at 38°C. for 2 hours.

4. Readings of agglutinations

Although tubes showing any degree of agglutination were considered as positive in the series of approximately 13,000 tests done at this laboratory, for finer differentiation many workers prefer to read the degree of positivity in each tube as I, II, III, IV. Weiner (14) has listed the following criteria:

IV. Massive agglutination of all or nearly all cells.

III. Large clumps with clear surrounding fluid.

II. Many smaller clumps with pinkness of surrounding fluid.

I. Minimal, but definite, agglutination visible.

The titer is then reported as the highest dilution in which any definite agglutination is visible with a magnifying lens against a strong light source. For this purpose a Kahn viewer gives excellent results.

III. PROPOSED METHOD FOR DETERMINATION OF COLD ISO-HEMAGGLUTININS

A. Venous Blood Sample - Obtaining Serum

1. Fasting blood is preferable in order to eliminate lipid particles.

2. 5 ml. are drawn into a chemically clean test tube.

3. Heparinized or oxalated plasma or serum may be used.

4. Hemolysis should be avoided.

5. Clotting.

a. The test tube rack is placed in a water bath at 37°C. for 1 hour.

b. The tubes are then centrifuged at 2000 R. P. M. for 30 minutes.

c. Serum may be decanted or plasma drawn up by bulb pipette.

B. Serum-Saline Titration.

1. The required number of tubes to be used for titration is placed in a Kahn rack. 13 x 100 mm. test tubes are used.

2. Omitting tube 1, 0.5 ml. of 0.85 per cent sodium chloride is placed in each tube thereafter.

3. 0.5 ml. quantities of serum are added to tubes 1 and 2. The serum and saline in tube 2 is mixed by repeatedly drawing up 0.4 ml. amounts into a 1 ml. pipette. 0.5 ml. of this serum-saline mixture is then transferred to tube 3 and mixed as in tube 2. This procedure is repeated in each tube thereafter. 0.5 ml. of solution is removed from the last tube and discarded. A final quantity of 0.5 ml. of solution remains in each tube.

C. Red Blood Cell Suspension

1. Type "O" donors should be used, preferably the same donor in all tests.

2. A 1% suspension in normal saline is prepared as follows:

a. The requisite volume of venous blood is drawn into a large test tube containing saline and mixed by inverting the tube four or five times.

b. The tubes are centrifuged at 1500 R.P.M. for ten minutes. The saline is decanted following which more saline is added. This procedure is repeated three times. The saline-red cell suspension is re-suspended in the desired final volume.

3. A fresh red cell suspension is used daily.

4. 0.5 ml. of a previously well shaken red cell suspension is added to each test tube in the rack. Each test tube now has 1 c.c. of solution. The solutions are mixed well by shaking. The final serum dilution in the first tube is now 1:2, in the second tube 1:4, etc.

D. Refrigeration

1. The racks are placed in a refrigerator at 2^{o} to 5^{o}C. If a large refrigerator (65 cu. ft.) is available, the temperature should be kept as constant as possible.

2. The racks should be left in a refrigerator overnight or from 12-16 hours. It is not necessary to have racks in a water-ice bath while in the refrigerator.

E. Reading

1. A good bright source of visible light and a stationary magnifying lens are used. A Kahn viewer is an excellent device for this purpose.

2. Shaking tubes:

a. By hand - the tests should be read close to the refrigerator. The rack is removed and the tubes are read immediately by gentle shaking sufficient to disperse red cells into solution. Vigorous shaking will result in lower titers and an increase of negative agglutinations.

b. By mechanical means — shortly before reading, racks are placed in a shaking machine and shaken for 4 seconds at 350 1-inch excursions per minute. They are immediately removed and tubes read without further shaking.

3. The highest dilution in which any degree of agglutination is visible is considered the titer of test.

IV. CONCLUSIONS

Observations were made on the present methods employed in the determination of cold hemagglutinins. Special attention was given to the use of fresh type "O" red blood cells daily, the careful pipetting of the serum-saline dilutions, and the reading of the tubes for red cell agglutination. A mechanical shaker obviated the subjective variant of manual shaking.

To obtain accurate reproducibility in this procedure, scrupulous attention must be given to small details and such details fully described when presenting the data.

V. RECOMMENDATIONS

A detailed method for determining the titer of cold hemagglutinins is presented with the hope that existing differences in technique between laboratories can be eliminated.

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